Induction of cyclo-oxygenase synthesis in human promyelocytic leukaemia (HL-60) cells during monocytic or granulocytic differentiation

Atsushi HONDA,*† Amiram RAZ† and Philip NEEDLEMAN†

*Department of Biochemistry, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan, and †Department of Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

Cyclo-oxygenase (COX) production in human promyelocytic leukaemia (HL-60) cells was studied during monocytic differentiation induced by $1\alpha,25$ -dihydroxyvitamin D_3 (24 nM; 3 days) or phorbol 12-myristate 13-acetate (100 nM; 1 day), or during granulocytic differentiation induced by retinoic acid (1 μ M; 4 days). Undifferentiated or differentiated HL-60 cells were labelled with [35 S]methionine, and membrane-bound COX was solubilized and quantified by SDS/PAGE. Immunoprecipitated 35 S-labelled COX from cells induced to differentiate into monocytic or granulocytic lineage were clearly detected on the autoradiograms as a protein of approx. 70 kDa molecular size, whereas only a very faint COX band was detected in untreated HL-60 cells. During both monocytic and granulocytic differentiation, COX activity (measured by the conversion of exogenous arachidonic acid into prostaglandin E_2) was dramatically increased. In addition, thromboxane synthesis was preferentially enhanced during monocytic differentiation. HL-60 cells, induced to differentiate into the monocytic or granulocytic lineage, provide a useful tool to investigate the cellular mechanisms involved in regulation of the synthesis of individual prostanoid-metabolizing enzymes.

INTRODUCTION

A promyelocytic leukaemia cell line, HL-60, is known to have the bipotential ability to differentiate either to monocyte-macrophage-like cells by exposure to either $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25$ -(OH) $_2D_3$; $1\alpha,25$ -dihydroxycholecalciferol] [1–3] or phorbol 12-myristate 13-acetate (PMA) [4], or to more mature myeloid cells (granulocytes) by exposure to dimethyl sulphoxide (DMSO) [5] or retinoic acid [6]. We and other investigators reported an enhancement of both thromboxane (Tx) and PGE $_2$ synthesis in HL-60 cells after monocytic differentiation induced by either $1\alpha,25$ -(OH) $_2D_3$ [7,8] or PMA [9], and in cells after granulocytic differentiation induced by DMSO [10,11] or retinoic acid [7]. The present studies were designed to investigate whether the increased prostaglandin production results from induction of *de novo* cyclo-oxygenase synthesis during differentiation. For this purpose, control and differentiated cells were labelled with [35 S]methionine and the rate of synthesis of newly formed COX *de novo* was assessed using selective immunoprecipitation with a polyclonal antibody raised against sheep seminal-vesicle COX. We found that differentiation of HL-60 cells along both monocytic and granulocytic lineage leads to a 5–7-fold increase in COX activity, which correlates with the production of newly synthesized COX. Furthermore, in monocytic cells, thromboxane synthase activity is also significantly increased.

MATERIALS AND METHODS

Materials

The following commercial materials were used: fetal-calf serum (FCS) from Grand Island Biological Co., Grand Island, NY, U.S.A.; human transferrin, all-*trans*-retinoic acid (all-*trans*-vitamin A acid; type XX), PMA ('TPA'), and goat anti-rabbit

IgG from Sigma Chemical Co., St. Louis, MO, U.S.A.; arachidonic acid from Nucheck, Elysian, MN, U.S.A.; purified sheep COX from Oxford Biomedicals, Oxford, MI, U.S.A.; Trans 35 S-label ([35 S]methionine; 5 mCi/0.451 ml; 1000 Ci/mmol; containing up to 15% [35 S]cysteine) from ICN Biomedicals, Irvine, CA, U.S.A.; methylated 14 C-labelled proteins (carbonic anhydrase, ovalbumin, albumin and phosphorylase *b*) for molecular-mass markers, and En 3 hance from du Pont–New England Nuclear, Boston, MA, U.S.A.; *Staphylococcus aureus* immunoprecipitin from Bethesda Research Laboratories; α_2 -macroglobulin from Boehringer Mannheim. $1\alpha,25$ -(OH) $_2D_3$ was kindly provided by Teijin Institute for Bio-Medical Research and Taisho Pharmaceutical Co., Tokyo, Japan. Polyclonal antibody against sheep seminal-vesicle COX was produced as described previously [12].

HL-60-cell cultures and cell differentiation

Human promyelocytic leukaemia cells (HL-60) were kindly supplied by Dr. S. Teitelbaum (Jewish Hospital, Washington University Medical Center, St. Louis, MO, U.S.A.). HL-60 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μ g/ml) at 37 °C in a humidified atmosphere of CO $_2$ /O $_2$ (1:19) as described in [7]. The cells were inoculated at about 6×10^5 cells/ml in 25 cm 2 T-flasks, and cell differentiation was induced by adding PMA (100 nM) or retinoic acid (1 μ M) in 10% FCS–RPMI 1640 medium for 1-day or 4-day culture periods respectively without a change of medium. $1\alpha,25$ -(OH) $_2D_3$ -induced monocytic differentiation of HL-60 cells was achieved by culturing cells (about 6×10^5 cells/ml) in serum-free Iscove's modified Dulbecco's medium (IMDM) containing 2 mM-L-glutamine, BSA (5 mg/ml), human transferrin (5 μ g/ml) and antibiotics (penicillin and streptomycin) [8] in 25 cm 2 T-flasks for

Abbreviations used: COX, cyclo-oxygenase; $1\alpha,25$ -(OH) $_2D_3$, $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25$ -dihydroxycholecalciferol); PGE $_2$, prostaglandin E_2 ; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulphoxide; FCS, fetal-calf serum; IMDM, Iscove's modified Dulbecco's medium; TxB $_2$, thromboxane B $_2$; DMEM, Dulbecco's modified Eagle's medium.

† To whom correspondence and reprint requests should be sent.

3 days without a change of medium (medium A) to exclude involvement of serum vitamin D-binding proteins. $1\alpha,25\text{-(OH)}_2\text{D}_3$ dissolved in ethanol was added to the IMDM medium to keep the final ethanol concentration less than 0.1 %, and the same amount of vehicle was added to the control cultures. At the end of each incubation, aliquots of the medium were assayed for prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2) production by specific radioimmunoassays [13].

Labelling of cellular proteins with [^{35}S]methionine

PMA- or $1\alpha,25\text{-(OH)}_2\text{D}_3$ -differentiated attaching cells were detached by forceful pipetting [7] and collected by centrifugation. Undifferentiated cells or cells treated with retinoic acid were similarly isolated by centrifugation. The cells were then washed with Dulbecco's modified Eagle's medium (DMEM) and incubated in 1 ml of DMEM with $10\text{ }\mu\text{M}$ -arachidonic acid at 37°C for 5 min. After incubation, the media were collected by centrifugation and assayed for production of PGE_2 and TxB_2 . The cells were then washed with methionine-poor DMEM (19 parts methionine-free DMEM to 1 part DMEM), and further incubated in 1 ml of methionine-poor DMEM containing $300\text{ }\mu\text{Ci}$ of Trans ^{35}S -label. After 4 h of labelling at 37°C , the medium was removed and the cells quickly washed with 1 ml of $\text{Ca}^{2+} + \text{Mg}^{2+}$ -free phosphate-buffered saline (25 mM-potassium phosphate/150 mM-NaCl, pH 7.4). The cells were then centrifuged and cell pellets kept at -30°C until further processed. For immunoprecipitation, cell pellets were sonicated in $150\text{ }\mu\text{l}$ of solubilization buffer [50 mM-Tris/1 mM-sodium *NN*-diethylthiocarbamate/10 mM-EDTA/1 % Tween-20/ α_2 -macroglobulin (0.2 mg/ml), pH 8.0] which effectively solubilized the microsomal COX. The sonicated material was centrifuged at 100000 g for 10 min to precipitate residual unsolubilized cellular membranes and the supernatant used for immunoprecipitation.

Staphylococcus aureus immunoprecipitation of ^{35}S -labelled COX

Purification and quantification of ^{35}S -labelled COX from induced or uninduced HL-60 cells were done by *S. aureus* immunoprecipitation, PAGE and fluorography as described by us previously [12]. Under these assay conditions, $>95\%$ of ^{35}S -labelled COX and $>90\%$ of the enzyme activity was immunoprecipitated from the sonicated HL-60 cells (determined as described in [12]; Fig. 1 below). After electrophoresis, the gels were fixed in methanol/water/acetic acid (4:5:1, by vol.) and then soaked in either 1 M-sodium salicylate for 10 min or Enhance (autoradiography enhancer) for 1 h, washed, dried, and exposed to Kodak XAR-5 X-ray film. Positions of the

various radioactive bands were calibrated using ^{14}C -labelled standard proteins. In some immunoprecipitation assays unlabelled pure sheep COX ($1\text{ }\mu\text{g/ml}$ of extraction buffer) was added to parallel aliquots ($30\text{ }\mu\text{l}$) of ^{35}S -labelled sonicated cells, and the mixture was subjected to immunoprecipitation and SDS/PAGE as described above.

Assay of COX activity

Cellular COX activity was determined after differentiation of the cells with the various agents employed. After washing of the cells, they were reincubated at 37°C for 5 min in medium containing arachidonic acid at a final concentration of $10\text{ }\mu\text{M}$, and the production of TxB_2 and PGE_2 was determined by specific radioimmunoassays [13,14]. Each antiserum against the specific prostanoid showed only negligible cross-reactivities ($<0.025\%$) against the other major prostanoids (PGE_2 , TxB_2 , 6-oxo-PGF $_{1\alpha}$ as applicable; see [15]).

Statistics

A probability level of $P < 0.05$ was considered significant when results were subjected to Student's *t* test.

RESULTS AND DISCUSSION

PG production and COX activity in HL-60 cells during monocytic or granulocytic differentiation

PG production and COX activity were determined in untreated HL-60 cells and in cells incubated with $1\alpha,25\text{-(OH)}_2\text{D}_3$, PMA or retinoic acid. After treatment with $1\alpha,25\text{-(OH)}_2\text{D}_3$ (24 nM), concentrations of both TxB_2 and PGE_2 in the culture medium increased gradually with incubation time, reaching approx. 40-fold and 3-fold stimulation respectively after 3 days in culture (Fig. 1a). In the same experiments, the COX activity, as estimated from the production rate of PGE_2 from added arachidonate, was increased about 27-fold, whereas TxB_2 production was increased 51-fold (Fig. 1b). These results suggest that, in addition to COX, Tx synthase may also be enhanced after monocytic differentiation with $1\alpha,25\text{-(OH)}_2\text{D}_3$, possibly via stimulation of synthesis of the enzyme *de novo*. This conclusion is in agreement with the results of Honda *et al.* [7], who showed that the Tx synthase activity is increased 2.5-fold in $1\alpha,25\text{-(OH)}_2\text{D}_3$ -induced cells.

In monocytic cells induced by treatment with PMA for 1 day, the production rates of TxB_2 and PGE_2 from arachidonate were very different, being approx. 32-fold higher for TxB_2 , but only 2-fold higher for PGE_2 when compared with uninduced cells

Table 1. PG production and COX activity in monocytic or granulocytic differentiated cells and in undifferentiated HL-60 cells

HL-60 cells were differentiated to monocytic cells by 1 day's exposure to PMA (100 nM) or to granulocytic cells by exposure to retinoic acid ($1\text{ }\mu\text{M}$; 4 days). TxB_2 and PGE_2 accumulated in culture medium during differentiation were radioimmunoassayed. Thereafter induced or uninduced cells were incubated in DMEM in the presence of exogenous arachidonic acid ($10\text{ }\mu\text{M}$) for 5 min at 37°C , and COX activity was assessed from the production rates of TxB_2 and PGE_2 from exogenous arachidonic acid. Values represent means $\pm$ S.D. for three separate flasks. Significant differences from values obtained with untreated cells: $^aP < 0.001$; $^bP < 0.01$; $^cP < 0.05$.

Treatment	PG production during differentiation period (ng/day per ml per 10^7 cells per culture)		COX activity (ng/5 min per ml per 10^7 cells)	
	TxB_2	PGE_2	TxB_2	PGE_2
None	4.6 ± 0.1	3.6 ± 0.3	2.1 ± 0.2	4.0 ± 0.6
PMA	7.9 ± 0.5^a	7.0 ± 0.5^b	67.1 ± 2.7^a	8.9 ± 0.3^a
None	1.5 ± 0.1	1.2 ± 0.1	0.6 ± 0.1	1.1 ± 0.1
Retinoic acid	2.2 ± 0.4^c	1.4 ± 0.1	3.7 ± 0.2^a	22.3 ± 1.9^a

Fig. 1. Time course of PG production and COX activity in HL-60 cells induced by $1\alpha,25-(\text{OH})_2\text{D}_3$

HL-60 cells were cultured for 1, 2 or 3 days in IMDM containing 0.6% (w/v) BSA and human transferrin (5 $\mu\text{g}/\text{ml}$) with (—) or without (---) $1\alpha,25-(\text{OH})_2\text{D}_3$ (24 nM) as described in the legend to Fig. 3, and amounts of prostaglandins accumulated in culture medium were determined (a). After that the cells were washed and further incubated in DMEM in the presence of exogenous arachidonic acid (10 μM) for 5 min at 37 °C, and the production rates of TxB_2 (●, ○) and PGE_2 (▲, △) were determined. Each point represents the mean $\pm$ s.d. from three separate experiments. Significant differences from values for untreated cells: *** P < 0.001; ** P < 0.01; * P < 0.05.

Fig. 2. COX production in HL-60 cells during monocytic differentiation induced by $1\alpha,25-(\text{OH})_2\text{D}_3$ or PMA or during granulocytic differentiation induced by retinoic acid

Untreated or differentiated HL-60 cells were labelled with [³⁵S]methionine for 4 h and the resulting solubilized sonicated cells were subsequently subjected to immunoprecipitation and SDS/PAGE. ³⁵S-labelled COX was immunoprecipitated from uninduced sonicated HL-60 cells (lanes 1, 3 and 5) or from cells induced with $1\alpha,25-(\text{OH})_2\text{D}_3$ (D; 48 nM, 3-day culture; lane 2), PMA (PM, 100 nM; 1-day culture, lane 4) or retinoic acid (RA, 1 μM ; 4 days; lane 6). ¹⁴C-labelled protein standards (Stds; lane 7) were BSA and ovalbumin (OVA).

(Table 1). These results suggest that in PMA-treated cells there is, in addition to stimulation of COX, also stimulation of thromboxane synthase activity. These findings and tentative conclusions are in agreement with a previous report [16] showing stimulation of both COX and thromboxane synthase activities in PMA-differentiated cells. Furthermore, we found that HL-60 cells induced to differentiate along the granulocytic lineage with retinoic acid also showed increased activity of COX and increased formation of TxB_2 . These results differ from those of Goerig and co-workers [16], who found no stimulation of COX or thromboxane synthase activities in HL-60 cells differentiated into neutrophils with DMSO.

COX synthesis in HL-60 cells during monocytic or granulocytic differentiation

In order to determine whether the increased levels of COX activity in differentiated cells were due to a higher rate of COX synthesis, we quantified by immunoprecipitation the synthetic rate of newly formed ³⁵S-labelled COX in untreated and in differentiated cells. In monocytic cells, induced by either $1\alpha,25-(\text{OH})_2\text{D}_3$ or PMA, and also in granulocytic cells induced by retinoic acid, a very marked COX band with a molecular size of approx. 70 kDa was seen, whereas only a very faint band was detected in untreated cells (Fig. 2). Quantification of the COX band intensities by densitometry indicated 23–25-fold stimulation of [³⁵S]COX synthesis in monocytic-differentiated cells [with $1\alpha,25-(\text{OH})_2\text{D}_3$] and an 11–23-fold stimulation in granulocytic-differentiated cells (with retinoic acid). With $1\alpha,25-(\text{OH})_2\text{D}_3$ as the inducing agent, a 2–3-day treatment period was required in order to detect substantial increase in synthesis *de novo* of COX (Fig. 3). The immunoselectivity of the anti-COX serum we employed in these studies was validated by demonstrating the ability of unlabelled COX to displace ³⁵S-labelled COX during immunoprecipitation. Fig. 4 shows that unlabelled COX (1 μg) displaced only the ³⁵S-labelled COX band (approx. mass of

Fig. 3. Time course of COX synthesis in HL-60 cells induced by $1\alpha,25-(\text{OH})_2\text{D}_3$

HL-60 cells were cultured for 1, 2 and 3 days as described in the legend to Fig. 1, with (+ D₃) (lanes 4–6) or without (– D₃; lanes 1–3) $1\alpha,25-(\text{OH})_2\text{D}_3$ (24 nM). The cells were then labelled with [³⁵S]methionine for 4 h and subsequently submitted to immunoprecipitation and SDS/PAGE. For other details, see the legend to Fig. 2.

70 kDa) without affecting the immunoprecipitation of other non-specific bands. The same results were obtained in PMA-induced monocytic cells (results not shown).

Overall, the data presented here suggest that differentiated HL-60 cells acquire increased activity and mass of the COX

Fig. 4. Displacement of ^{35}S -labelled COX by unlabelled enzyme

Aliquots of ^{35}S -labelled sonicated cells with (+) or without (–) sheep COX (1 μg) were immunoprecipitated and subjected to SDS/PAGE. Results are shown for ^{35}S -labelled COX isolated from $1\alpha,25\text{-(OH)}_2\text{D}_3$ -induced cells (D_3 ; lanes 2 and 3) and for retinoic acid-induced cells (RA; lanes 4 and 5). Conditions for differentiation of cells were as described in the legend to Fig. 2. ^{14}C -labelled protein standards (Stds; lane 1) are as given in the legend to Fig. 2. * denotes non-specific bands.

enzyme, the extent of the increase depending on the type of differentiating agent employed. At least one other prostanoid enzyme, Tx synthase, appears to be stimulated under monocytic differentiation. Two recent reports [16,17] have shown that differentiation of HL-60 cells by DMSO into granulocytes is accompanied by a marked, 15–20-fold, increase in 5-lipoxygenase activity and leukotriene B_4 production. Furthermore, the two modes of differentiation also differ in their ability to up-regulate phospholipase A_2 after differentiation. Bosner *et al.* [10] showed that DMSO-differentiated granulocytes contain increased phospholipase A_2 activity, whereas Honda and co-workers [7], assaying lipase activity both by release of ^{14}C arachidonate from prelabelled cells and by direct measurement of phospholipase A_2 activity in cell homogenates, found no differences between undifferentiated and $1\alpha,25\text{-(OH)}_2\text{D}_3$ -differentiated cells. It thus appears that the differentiation of HL-60 cells into specific

monocytic-or granulocytic-lineage cells is accompanied by selective regulation of several key enzymes involved in the synthesis of PGs, Tx and leukotrienes. Further studies utilizing these varied differentiating cells could provide insight into the mechanism(s) for selective modulation of eicosanoid enzymes.

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